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A role for the C3a anaphylatoxin receptor in the effector phase of asthma

Alison A. Humbles*, Bao Lu*, Christy A. Nilsson*, Craig Lilly†, Elliot Israel†, Yuko Fujiwara‡, Norma P. Gerard* & Craig Gerard*

* Ina Sue Perlmutter Laboratory, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA
† Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA
‡ Division of Hematology and Oncology, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

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Asthma is a chronic inflammatory disease of the airways and lung mucosa with a strong correlation to atopy and acquired (IgE) immunity[1]. However, many features of bronchial asthma, such as smooth muscle contraction, mucus secretion and recruitment of inflammatory cells, are consistent with the actions of complement anaphylatoxins, in particular C3a and C5a[2]. Complement activation forms a central core of innate immune defence against mucosal bacteria, viruses, fungi, helminths and other pathogens. As a system of 'pattern-recognition molecules', foreign surface antigens and immune complexes lead to a proteolytic cascade culminating in a lytic membrane attack[2,3]. The anaphylatoxins C3a and C5a are liberated as activation byproducts and are potent pro-inflammatory mediators that bind to specific cell surface receptors and cause leukocyte activation, smooth muscle contraction and vascular permeability[2]. Here we show that in a murine model of allergic airway disease, genetic deletion of the C3a receptor protects against the changes in lung physiology seen after allergen challenge. Furthermore, human asthmatics develop significant levels of ligand C3a following intra-pulmonary deposition of allergen, but not saline. We propose that, in addition to acquired immune responses, the innate immune system and complement (C3a in particular) are involved in the pathogenesis of asthma.

To investigate the role of the anaphylatoxins in host defence and inflammation, we have generated mouse strains deficient in the receptors for C5a and C3a. Mice lacking the C5a receptor have altered lung mucosal defence against pseudomonas pneumonia[4], and have tissue-variable protection against injury mediated by immune complexes[5]. Additionally, evidence for a 'protective' role of C5a in models of brain[6] and pancreatic inflammation[7] have been observed using these mice, indicating that the C5a receptor may be able to modulate both pro- and anti-inflammatory effects.

The C3a receptor (C3aR) is an unusual member of the peptidergic G-protein-coupled receptor family: it has a large extracellular loop (172 amino acids) between transmembrane segments 4 and 5 (refs 8, 9). Although the bulk of this loop may be mutated without affecting C3a function at the receptor, the conservation of this unusual feature across species suggests the potential for an alternative ligand[10]. Mice have been generated that are deficient in the ligand precursor C3, and thus lack C3a, but these mice are also deficient in the other C3-derived biological activities (for example, phagocytosis and regulation of B-cell function of T-cell-dependent antigens independent of the C3a receptor)[11]. We generated C3aR homozygous knockout mice on a BALB/c and 129/sv mixed background by homologous recombination with standard techniques. Figure 1 shows the construct, detection and function of the mutant alleles. C3aR-deficient mice were born at the expected mendelian ratios, showed no overt developmental or morphological abnormalities and were fertile when maintained under specific pathogen-free conditions.

Although the function of C3a in disease is not as well defined as that of C5a, there is evidence that C3a/C3aR is involved in allergic disease: C3a has been shown to activate and be chemotactic for human eosinophils and mast cells[12-14], and to contract human lung parenchymal strips[15]. Therefore, the presence of this receptor on airway smooth muscle and cells associated with allergic disease prompted us to investigate its role in a mouse model of allergic asthma. We used a standard model involving low-dose immunization with ovalbumin (OA) and aluminium hydroxide followed by serial aerosol challenge on days 21–24 to generate allergic airway disease in mice backcrossed three generations against the BALB/c strain. This strain of mouse is routinely used in this model because of the response seen in eosinophilic airway inflammation, Th-2 cytokine production and airway hyper-responsiveness. At both 6 and 24 h after the last aerosol challenge, both wild-type and C3aR-deficient mice developed a similar lung inflammatory infiltrate as assessed by analysis of total white blood cells in the bronchoalveolar lavage (BAL) (Fig. 2a). Interestingly, there was no difference in eosinophil numbers in either the BAL or lung tissue of wild-type versus C3aR-deficient mice when using this repeated OA-challenge protocol (Fig. 2b). IgE levels were also identical and there were no differences in the BAL fluid levels of interleukin (IL)-4, IL-5 and IL-13 (not shown).

We used whole body plethysmography to measure the physiological response of sensitized and sham-treated mice to aerosolized methacholine. Airway hyper-responsiveness (AHR) was measured 24 h after the last aerosol challenge by recording respiratory pressure curves, and was expressed as enhanced pause (Penh), a calculated value that correlates with measurement of airway resistance, obstruction and intrapleural pressure in the same mouse[16]. As shown in Fig. 3, when sham-treated wild-type littermates are compared with sensitized/challenged wild-type littermates, airway hyper-responsiveness to inhaled methacholine is markedly enhanced. In contrast, when C3aR-deficient mice are treated identically, there is no statistically significant change in airway responsiveness to methacholine. Therefore, absence of C3aR confers significant protection against increased AHR. Using the same sensitization and challenge protocol, we assessed AHR to methacholine in anaesthetized, tracheostomized, mechanically ventilated mice. This system allows the simultaneous measurement of pul-

monary conductance (GL) and compliance (Cdyn) in response to intravenous infusion of a bronchoconstrictor[17]. Concomitantly, using two parameters to assess lung function, we found that again sensitized/challenged C3aR-deficient mice were protected from increased AHR to methacholine (Fig. 4). Interestingly, this effect was more pronounced with respect to pulmonary compliance (Fig. 4, right panel).

Because of previous reports associating complement activation with some patients with exercise-induced asthma[18] and status asthmaticus[19], we also measured C3a levels in lung lavage fluids of
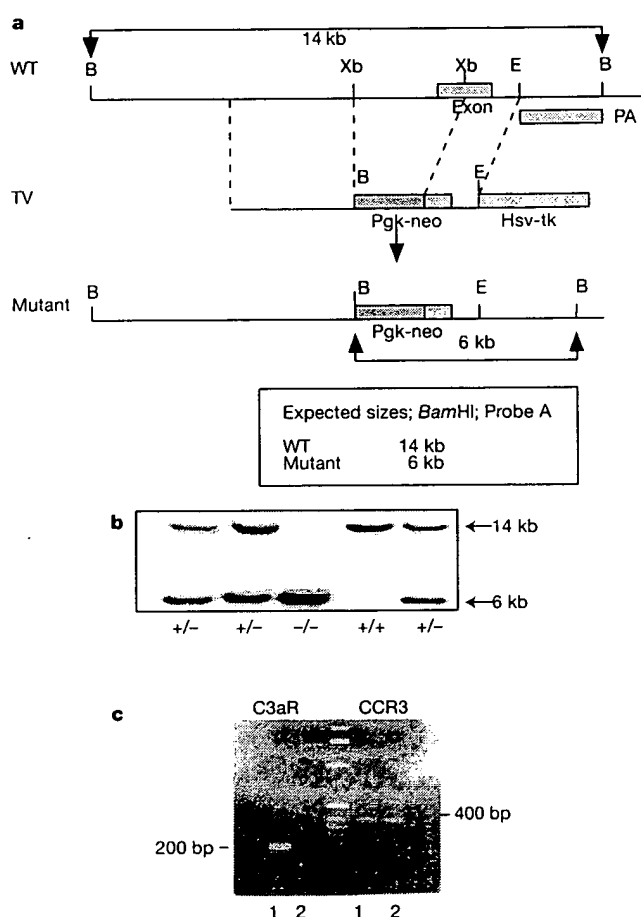

Figure 1 Targeted disruption of the mouse C3aR gene. **a**, Targeting strategy. Wild-type (WT) allele (top), targeting vector (TV: middle) and predicted mutated allele (bottom). The wild-type gene is shown containing a single exon of the receptor as an open block bounded by two BamHI sites. Around 736 base pairs of the N-terminal region including the start codon was deleted and replaced with a neomycin-resistant gene driven by the PGK promoter. Homologous recombination led to a diagnostic pattern on restriction digests probed with a downstream flanking sequence (Probe A/PA). B, BamHI; E, EcoRI; Xb, XbaI. **b**, Representative Southern blot analysis of tail DNA from littermates. Genomic DNA was digested with BamHI and hybridized with flanking probe A. The 14-kb wild-type and 6-kb targeted allele BamHI fragments identified by probe A are shown. **c**, Polymerase chain reaction with reverse transcriptase (RT−PCR) analysis of C3aR expression using total RNA from bone marrow cells of wild-type and homozygote littermates. RT−PCR was also performed using primers for CCR3 messenger RNA as a control for the presence of amplifiable RNA.
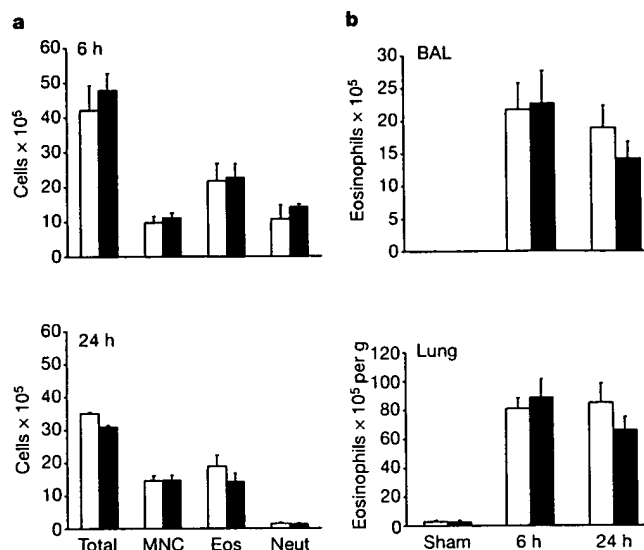

Figure 2 Pulmonary accumulation of inflammatory cells. Sham or sensitized mice were exposed to saline or OA aerosol on days 21−24. **a**, Total cell numbers in BAL from C3aR-deficient (solid bars) or wild-type littermate (open bars) mice were assessed 6 (top) and 24 h (bottom) after the last aerosol challenge. MNC, mononuclear cells; Eos, eosinophils; Neut, neutrophils. **b**, Eosinophil numbers in BAL (top) or lung tissue (bottom) at 6 and 24 h after allergen challenge. Results are presented as means $\pm$ s.e.m. (sham, $n = 8$; 6 h, $n = 10-12$ mice per group; 24 h: $n = 26-27$ mice per group).
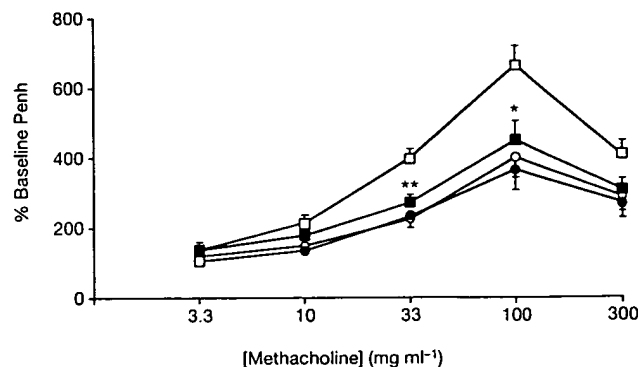

Figure 3 Assessment of AHR to inhaled methacholine (MCh) in conscious mice. Sham-treated wild-type (open circles) or C3aR-deficient (filled circles) mice were exposed to aerosolized saline, and OA-sensitized wild-type (open squares) or C3aR-deficient (filled squares) mice were exposed to aerosolized OA on days 21−24. Airway responses to increasing concentrations of MCh were assessed 24 h after the last aerosol exposure using barometric whole-body plethysmography. The dose−response curves of MCh-induced increases in Penh are shown. Results are expressed as the means $\pm$ s.e.m. (sham, $n = 12$ mice per group; sensitized/OA challenged, $n = 20-22$ mice per group) of the per cent increase in Penh compared with the baseline Penh values after saline exposure. Significant differences between sensitized/challenged wild-type and C3aR-deficient mice are indicated as $^*P < 0.01$ and $^{**}P < 0.001$, as determined by unpaired Student's t-test.

asthmatic subjects after segmental allergen challenge. Eight atopic asthmatics underwent bronchoscopy and intrapulmonary challenge with allergen diluent (sham) in one lobe and with allergen, to which they exhibited a positive skin test and a 20% reduction in FEV_1 (the forced expiratory volume in the first second), in the other lobe. Four to eight hours later, at the time of onset of the late-phase response in these subjects, the same lobes were lavaged with saline at repeat bronchoscopy. As an added control, five non-asthmatic adults were lavaged with saline alone. Samples of BAL from all subjects were assayed for C3a/C3a des-Arginine by radioimmunoassay (RIA). As shown in Table 1, we saw a significant increase in C3a anaphylatoxin in the allergen-challenged lobes compared with the sham-treated lobes when the results were expressed as median values with interquartile ranges (IQR; $P < 0.01$). Significantly, as the levels of C3a in normal subjects (non-asthmatics) and sham-challenged asthmatics were identical, it is unlikely that trauma during broncho-scopy induced complement activation, leading to C3 generation. We also found that anaphylatoxin formation in asthmatic subjects was positively correlated with neutrophil numbers in BAL ($R^2 = 0.9$, $P < 0.01$, data not shown).

For the purpose of understanding its mechanism, asthma may be divided into three phases: initiation, propagation and effector. The initiating phase in atopic asthma is believed to involve IgE and mast cells[20], whereas this phase in cold-air or exercise-induced asthma is less well understood. In the initiating phase, genetic predispostion to atopy predominates. Polygenic traits involving IL-4, IL-5, IL-13 and the development of IgE antibodies against environmental antigens occur. In the propagation phase, Th-2-polarized T lymphocytes and eosinophils infiltrate the airways, leading to a chronic inflammatory state[21]. In the effector phase, elaboration of spasmogenic substances and secretogogues cause bronchoconstriction and mucus hypersecretion. Effective therapies against the effector phase of asthma have been developed. Arachidonic acid metabolites centred on the cysteinyl leukotrienes are blocked by 5-lipoxygenase inhibitors and leukotriene receptor antagonists, and these drugs are effective in some patients[22]. However, a significant number of

asthma patients are refractory to these therapies, indicating that parallel pathways may generate other spasmogenic mediators. A number of mediators have been suggested as participating in the effector phase of asthma, including neuropeptides and endothelins[23,24]. Here, we identify the C3a receptor in the mouse as a significant contributor to the effector phase of allergic bronchoconstriction. We additionally identify the ligand C3a in BAL fluids of allergen- but not sham-challenged human asthmatics during their late-phase response, 4–8 h after allergen delivery to the airway.

What mechanisms might lead to the generation of C3a *in vivo* in atopy and asthma? First, it is possible that in addition to activation of complement through the classical pathways with both IgE and IgG, some macromolecular structures from the dust mite, fungi or ragweed allergen may be recognized by the alternative pathway and fix C3 directly. Pattern recognition of carbohydrate structures by the mannose-binding lectin pathway is also conceivable. However, not all subjects wheeze when exposed to these potentially complement-activating stimuli. Another possibility is that mast cell proteases released directly after IgE engagement of allergen on mast cells/basophils could generate C3a directly, as occurs *in vitro*[25]. Thus, with C3 levels naturally high in interstitial and lung lining fluids, allergen-triggered release of mast cell proteases in atopic individuals could lead to C3a release without typical activation of the entire complement cascade. Direct anaphylatoxin receptor blockade, which is possible as it is a member of the G-protein-coupled receptor superfamily, might be therapeutic in some human atopic asthma patients. □

Methods

Targeted disruption of the mouse C3aR gene

We screened a mouse 129/sv genomic library with mouse C3aR complementary DNA. An 8-kilobase (kb) genomic fragment containing the C3aR gene was used to construct the targeting vector. We deleted a 3-kb *Xba*I fragment containing a partial amino-terminal and 5' untranslated region (approximately 736 base pairs) and replaced it with a neomycin-resistant gene driven by the PGK promoter. This mutated fragment was subcloned into the pPNT vector for double selection with geneticin and glancyclovir. The targeted vector was linearized and electroporated into J1 embryonic stem cells, and the correctly targeted event was screened by Southern blotting. We injected two targeted cell lines into blastocysts derived from C57BL/6 mice. Chimaeric males were bred with female BALC/c to yield germline transmission of the targeted allele. Heterozygote animals were then bred with wild-type BALB/c mice to give third generation BALB/c backcrossed mice. Heterozygote animals from these mice were then bred against each other to yield homozygote C3aR$^{-/-}$ and C3aR$^{+/+}$ mice, which were used for all studies.

Animal studies

These were performed according to institutional and NIH guidelines for animal use and care.

Immunization and challenge protocol

We immunized mice with 10 µg of OA and 1 mg of aluminium hydroxide, made up in 0.2 ml sterile saline and injected intraperitoneally on days 0, 7 and 14. Sham-immunized mice received aluminium hydroxide alone. On days 21–24, mice were exposed to aerosolized OA (5%) or saline for 40 min. They were killed with a barbiturate overdose 6 or 24 h after the last aerosol challenge and BAL was performed with 2 × 1-ml aliquots of HBSS containing 10 mM HEPES and 10 mM EDTA. We determined total cell numbers in BAL using kimura staining, and performed differential cell counts (of at least 500 cells per slide) on cytospin preparations stained with eosin and methylene blue (Diff-Quik, Dade Diagnostics). Eosinophil numbers in lung tissue were quantified by measuring eosinophil peroxidase as described[26].

Determination of AHR in conscious animals

AHR was measured in unrestrained conscious animals using barometric plethysmography[16] (Buxco Electronics). AHR is expressed as the fold increase in Penh (enhanced pause). Twenty-four hours after the last aerosol challenge, mice were placed in whole-body plethysmographic chambers and exposed for 1 min to aerosolized saline and subsequently to increasing concentrations of aerosolized methacholine (3.3–300 mg ml^{-1}). Recordings were taken for 10 min after each aerosolization. The Penh values measured during each 10-min cycle were averaged and expressed for each methacholine concentration as a percentage of the baseline Penh value following saline exposure.

Determination of AHR in anaesthetized animals

Each mouse was anaesthetized, a tracheostomy was created and mechanical ventilation was instituted through a tracheal cannula. We measured the pulmonary mechanical
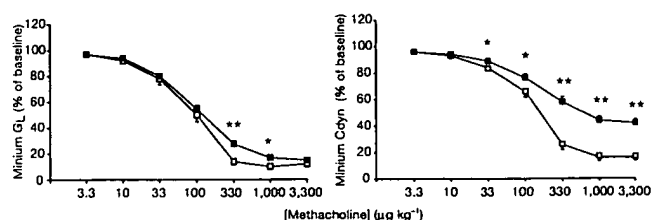

Figure 4 Airway responsiveness to intravenous methacholine (MCh) in anaesthetized mice. OA-sensitized wild-type (open squares) or C3aR-deficient (filled squares) mice were exposed to aerosolized OA on days 21–24. Twenty-four hours after the last aerosol exposure, mice were anaesthetized, intubated and mechanically ventilated, and airway responses to increasing doses of intravenous MCh assessed. The dose–response curves for pulmonary conductance (G_L, left) and pulmonary compliance (Cdyn, right) are shown. Results are expressed as the means ± s.e.m. (wild type, $n = 11$; C3aR$^{-/-}$, $n = 16$ mice) of the per cent minimal decrease in pulmonary conductance or compliance obtained after MCh challenge compared with the baseline value just before challenge. Significant differences between sensitized/challenged wild-type and C3aR$^{-/-}$ mice are indicated as $*P < 0.04–0.01$ and $**P < 0.001$ as determined by unpaired Student's t-test.

Table 1 C3a/C3a des-Arginine levels in human BAL fluid

	Normals (n = 5)	Asthmatics (n = 8)		
		Pre-allergen	Sham	Allergen
Median	59	64	69	123*
IQR	58–107	42–79	46–91	55–357

C3a/C3a des-Arginine levels (expressed in ng ml^{-1}) were measured in BAL fluid by radioimmunoassay. Results shown are the medians and the interquartile ranges (IQR) for five normal and eight asthmatic subjects that underwent segmental sham or allergen challenge.
*P < 0.01, significant difference between the sham and allergen groups, as determined by the Wilcoxin signed-ranks test.

parameters GL (pulmonary conductance) and Cdyn (pulmonary compliance) as described[17]. Logarithmically increasing doses of methacholine (3.3–3,300 µg kg^{-1}) were administered intravenously through a jugular venous catheter. Maximally reduced GL and Cdyn values after each methacholine dose were expressed as percentages of their values obtained just before the infusion of that dose. Intervals of 3–5 min were allowed to elaspe between doses to allow GL and Cdyn to return to within 10% of the baseline obtained before the preceding dose.

Segmental allergen challenge of human subjects

Approval for the human study was granted by the Brigham and Women's Hospital Review Board. The study subjects included five control subjects without any history of respiratory disorder, including asthma, and eight allergic asthmatics who met the American Thoracic Society criteria for an asthma diagnosis[27]. Each asthmatic subject had an abnormal allergen skin prick test defined as a weal of greater than 5 mm to 1 of 12 standard allergens in 50% vol/vol glycerin solution (Eastern 10-tree mix, ragweed mix, 3-weed mix, dog epithelium/mixed breeds, timothy grass, *Dermatopagoides farinae*, *D. pteronyssinus Alternaria tenius*, coakroach, *Cladosporium herbarum* and *Aspergillus* mix (all from Greer Laboratories), and cat hair standardized extract (ALK Laboratories)).

Before the study, the subjects were exposed to increasing concentrations of neubulized allergen to determine the concentration required to reduce their FEV$_1$ by 20%. We performed segmental allergen challenge with seasonal allergens at times when these agents were not in season. At this time all subjects were free of respiratory symptoms and had stable lung function for at least 4 weeks following the inhalation challenge before segmental challenge, for which procedure subjects underwent two serial bronchoscopic procedures 4–8 h apart.

Bronchoscopy was performed under midazolam/fentanyl conscious sedation. For normal and asthmatic pre-allergen samples, the bronchoscope was advanced to airway occlusion in the anterior segment of the left upper lobe and 3 × 50-ml aliquots of warmed sterile saline instilled and gently aspirated; in asthmatics only, the bronchoscope was then moved and advanced into the right upper lobe, where 2 ml of allergen diluent was delivered; after 5 min the bronchoscope was moved again and advanced into the right middle lobe, where 2 ml of allergen solution was delivered. The broncoscope was then removed, and focal wheezing was confirmed to be present exclusively over the right middle lobe in all subjects by auscultation. Four to eight hours later, a second bronchoscopy was performed to obtain BAL fluid from the right upper lobe (sham sample) and the right middle lobe (allergen sample).

Measurement of C3a/C3a des-Arginine levels in BAL fluid

BAL fluid samples were collected onto ice, strained through a gauze mesh and centrifuged (700g at 4 °C) and stored at −80 °C until assayed. C3a/C3a des-Arginine levels were measured using an RIA kit (Amersham Pharmacia Biotech). Interference from the precursor C3 in BAL samples was avoided by the inclusion of a selective precipitation step before the assay. All samples were assayed undiluted according to the protocol outlined by the manufacturers.

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A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite

Sanjay A. Desai*†, Sergey M. Bezrukov‡ & Joshua Zimmerberg†

* *The Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, ‡ The Laboratory of Physical and Structural Biology, and † The Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Development, National Institutes of Health, Bethesda, Maryland 20892, USA*

Growth of the malaria parasite in human red blood cells (RBCs) is accompanied by an increased uptake of many solutes including anions[1], sugars[2], purines[3], amino acids[4] and organic cations[5]. Although the pharmacological properties and selectivity of this uptake suggest that a chloride channel is involved, the precise mechanism has not been identified. Moreover, the location of this uptake in the infected RBC is unknown because tracer studies are complicated by possible uptake through fluid-phase pinocytosis[6] or membranous ducts[7]. Here we have studied the permeability of infected RBCs using the whole-cell voltage-clamp method. With this method, uninfected RBCs had ohmic whole-cell conductances of less than 100 pS, consistent with their low tracer permeabilities[8]. In contrast, trophozoite-infected RBCs exhibited voltage-dependent, non-saturating currents that were 150-fold larger, predominantly carried by anions and abruptly abolished by channel blockers. Patch-clamp measurements and spectral analysis confirmed that a small (< 10 pS) ion channel on the infected RBC surface, present at about 1,000 copies per cell, is responsible for these currents. Because its pharmacological properties and substrate selectivities match those seen with tracer studies, this channel accounts for the increased uptake of small solutes in infected RBCs. The surface location of this new channel